Original Article

SMO mutations confer poor prognosis in malignant pleural mesothelioma

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Background: Malignant pleural mesothelioma (MPM) is an aggressive tumor but approximately 12% of patients survive more than 3 years. The biological differences underlying better outcomes are not known. Several targeted agents and immunotherapy have been ineffective. Hedgehog (Hh) is one emerging pathway. We compared the biological profiles of patients with different survival, investigating the most frequently altered genes, including the Hh pathway.

Methods: We analyzed 56 MPM. A 36-month overall survival (OS) cut-off divided patients into 32 normo (NS) and 24 long (LS) survivors. We used next generation sequencing to test 21 genes, immunohistochemistry to evaluate SMO expression. Mutation differences between NS and LS and their associations with clinical features were analysed by Fisher's test, OS with the Kaplan-Meier method and its association with mutations by univariate and multivariate Cox proportional hazard models.

Results: Clinical features were similar in both groups. Eighteen out of 56 patients (32%) were wild-type for the genes analysed. At least five had mutations in *BAP1*, *NF2*, *TP53*, *SMO* and *PTCH1* with no significant differences between the groups except for *SMO*. *SMO*, a member of the Hh pathway, was mutated only in NS (15.6%) and only *SMO* mutations were significantly associated with poor prognosis at univariate (HR =4.36, 95% CI: 2.32–8.18, P<0.0001) and multivariate (HR =9.2, 95% CI: 3.0–28.4, P=0.0001) analysis. All *SMO* mutated patients expressed high protein levels.

Conclusions: *SMO* mutations were clearly associated with worse prognosis. SMO may be a therapeutic target but this needs to be confirmed in a prospective trial.

Keywords: Gene mutations; hedgehog pathway; malignant pleural mesothelioma (MPM); prognosis; *SMO*

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Introduction

Malignant pleural mesothelioma (MPM) is a rare cancer associated with asbestos exposure (1). Thus, incidence rates differ across countries. Although the use of asbestos has been banned in 55 countries for at least the last 20 years, about 125 million people worldwide are still exposed to it (2). On the basis of global asbestos consumption over the last decades, a further mesothelioma wave can be expected, involving large geographic areas (3), and may peak in developed countries by 2030 (4).

In the last few decades, the identification of specific molecular targets and genetic alterations has radically changed the treatment paradigms for different cancers, improving outcomes. Unfortunately, this is not the case for MPM patients, whose prognosis remains poor, with median survival of about 12 months (5). The roles of surgery and radiotherapy are debated (6). Since 2003, the only treatment that has slightly improved survival is platinum-based doublet with an antifolate agent (7,8). The association of the antiangiogenic drug bevacizumab to chemotherapy has been explored in several studies and was recently reported to give significant improvement of survival, although the clinical application of this association remains uncertain (9-11). Despite a consistent biological rationale and promising preclinical data, several targeted agents and immunotherapy with anti-CTLA4 have shown no efficacy in unselected patients. Other immune-checkpoint inhibitors and new-generation compounds are now under investigation (12,13).

In the single arm phase II MERIT trial, monotherapy with anti-PD-1 nivolumab was administered in 34 MPM patients as second- or third-line treatment; objective response rate (ORR), median progression-free survival (PFS) and overall survival (OS) were 29%, 6.1 and 17.3 months, respectively (14). Based on these results, nivolumab was approved in Japan for unresectable recurrent MPM. Other phase II studies demonstrated a potential activity of nivolumab in MPM (15). However, in a randomized phase III trial the anti-PD-1 pembrolizumab failed to show a PFS or OS benefit in advanced pretreated MPM patients in comparison with single agent chemotherapy (16). In MPM also the predictive role of PD-L1 expression for immunotherapy is still debatable (15).

Nevertheless, according to population studies, approximately 12% of MPM patients survive more than 3 years (17). Biological or molecular differences that might explain the better outcome of these longer-term survivors are still unknown. Histological subtype, age, and stage are recognized prognostic factors (18,19).

From the biological point of view, the main genetic alterations in MPM patients involve a handful of genes (i.e., *TP53*, *NF2*, *BAP1* and *CDKN2A*) (20-25). However, their real predictive or prognostic value is still uncertain (22-25). Furthermore, these alterations are not easily druggable and first attempts to target them have already failed (26).

Among the new pathways reported in MPM, Hedgehog (Hh) is emerging. Hh is involved in cell proliferation, survival, epithelial-to-mesenchymal transition, stemness and differentiation during embryonic development. Hh ligands bind to PTCH1 and PTCH2 receptors. If the ligand is absent, PTCH1 or PTCH2 binds to the SMO co-receptor, repressing its activity. Consequently, the transcription factors (GLI family) are not activated and transcription is stopped. In contrast, when the ligand binds to the receptor, the SMO repression is released, leading to transcriptional activation (*Figure 1*) (27). Normally the Hh pathway is inactive in adult tissues (28). Inappropriate reactivation of Hh signaling, mainly due to mutations in key pathway regulators (e.g., *PTCH*, *SUFU* or *SMO*) or over-expression of pathway activators (Hh ligands, *SMO*, *GLI1*, *GLI2*), has been linked to different sporadic malignancies, including basal cell carcinoma (BCC), pancreatic adenocarcinoma and gastrointestinal stromal tumors (GIST) (29,30). In particular a strong correlation between higher SMO, SHH, GLI expression levels and poorer OS was observed in MPM patients. High levels of Hh involved genes were detected in MPM compared to normal pleura (31,32). Furthermore, data available in public database at the beginning of the study reported *SMO* and *PTCH1* among the most frequent mutated genes in MPM (33). This study was designed to distinguish possible differences in the biological profiles of MPM patients with different survival periods, investigating the most frequently altered genes, including those in the Hh pathway.

We present the following article in accordance with the MDAR Reporting Checklist (available at http://dx.doi.org/10.21037/tlcr-19-425).

Methods

Selection of patients and sample collection

Following the evidence from our Italian epidemiologic study on pleural mesothelioma, LUME (LUngo sopravviventi nel MEsotelioma pleurico) (17), we established an OS cut-off of 36 months to divide patients into normo survivors

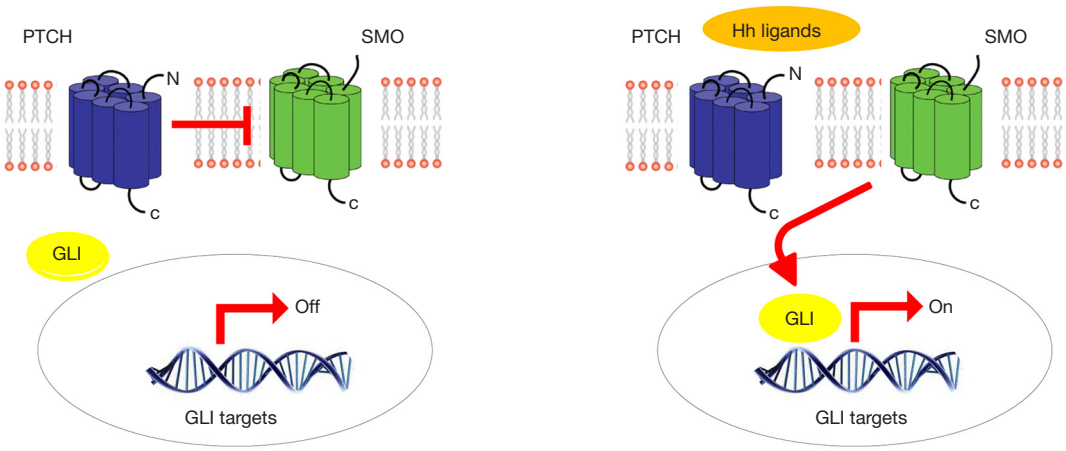

Figure 1 Hedgehog (Hh) ligands bind to PTCH receptors. If Hh ligands are absent, PTCH binds and represses SMO co-receptor. Thus, the transcription factors GLI are not activated and transcription is stopped. Conversely, when Hh ligands bind to PTCH, the SMO repression is released, leading to transcriptional activation. PTCH, patched homologue; SMO, smoothened protein; GLI, glioma-associated oncogene.

(NS) and long survivors (LS). This cut-off guarantees the identification of true LS, three times the median survival (5).

We designed a pilot study in which 60 patients (30 LS and 30 NS) had to be retrospectively enrolled. The recruitment period was 2002–2014 and three Italian institutions contributed: INT, Istituto Oncologico Veneto-Padua and Azienda Ospedaliera-Parma. We considered only patients with enough tissue samples to perform molecular tests and with almost all clinical and pathological data available. For each patient, clinical data (disease characteristics, surgical treatments and outcomes) and formalin-fixed paraffin-embedded (FFPE) tissue samples at diagnosis were collected. FFPE were centrally reviewed and analysed at the Fondazione IRCSS Istituto Nazionale dei Tumori-Milan (INT). We included in the study all the LS available and we randomly identified, from the remaining patients, the MPM NS to see whether they had a biological profile different from the MPM LS. No differences in clinical characteristics (i.e., stage, age, sex and treatment) were observed between MPM patients included and excluded from the study.

The present study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All the experimental protocols were approved by INT Independent Ethics Committee, code INT 91/13. Because the study was retrospective, the patients were not in treatment or in active follow-up; therefore, their informed consent was not required in accordance with Italian law (Gazzetta Ufficiale n. 72, 26/03/2012; n. 303, 29/12/2016).

Genomic DNA extraction and next generation sequencing (NGS)

FFPE sample was sliced in 5-µm sections and manually microdissected to isolate the highest percentage of neoplastic cells as possible. Sample was treated with xylene and 100% ethanol to remove paraffin and then DNA was isolated using the GeneRead DNA FFPE kit (Qiagen). DNA amount was quantified with Qbit dsDNA BR kit (ThermoFisher). gDNA (40 ng) was profiled using a customized panel (Ampliseq Designer, ThermoFisher) that amplify 21 genes (*CDKN2A*, *NF2*, *GSTM1*, *NAT2*, *BAP1*, *TERT*, *TP53*, *PTCH1*, *SMO*, *LATS2*, *KEAP1*, *PI3KCA*, *KRAS*, *NRAS*, *STK11*, *WT1*, *FBXW7*, *CTNNB1*, *KIT*, *KDR*, and *REV3*). Our genes selection was based on those reported in the public MPM database (33). The library was prepared by IonAmpliSeq Library kit 2.0 (ThermoFisher) according to the manufacturer's instructions. Emulsion PCR was performed on the Ion One Touch 2 instrument (ThermoFisher) using Ion PGM™ template OT2 200 kit, according to the manufacturer's instructions. Sequencing was carried out on the Ion PGM System (ThermoFisher) using Ion 318 v2 Chip and Ion PGM™ sequencing 200 kit v2 according to the manufacturer's instructions. Data was processed by using Torrent Suite SoftwareTM v4.4.2; the variant calling from sequencing data was generated by

Variant Caller plugin. The resulting variants was annotated using Ensemble Variant Effect Predictor pipeline, Ion Reporter™ analysis software, ClinVar db and COSMIC database. The filtered variants were examined using the Integrative Genomic Viewer IGV tool (34). The coverage depth was always more than 500× and the reported mutations had a frequency of at least 5%. Matched normal DNA was used for six patients; where normal tissue is not available, we filtering germline variants by using publically-available or proprietary database of known polymorphisms (e.g., dbSNP, ExAC, 1000 Genomes), excluding variant with minor allele frequency (MAF) $>10^{-6}$.

Immunohistochemistry (IHC)

IHC was done on FFPE whole-tissue sections. SMO mouse monoclonal antibody was purchased from Origene, TA318627, clone 3E5 and used at the dilution of 1:500. In accordance with the Ventana BenchMark ultra-automated system protocol, the antigen retrieval Ultra Cell Conditioning and standard reagents, provided by Ventana (OptiView DAB IHC Detection Kit; Ventana Medical Systems), were used. The staining intensity (I) and the percentage of positive cells (P) were evaluated by a trained pathologist and a semi-quantitative score S = I × P was calculated.

Statistical analysis

Gene mutation differences between NS and LS and the association between gene mutations and clinical features were assessed by the non-parametric Fisher's exact test. The *t*-test was used for the association of gene mutations with age. OS was analyzed from the date of diagnosis to the date of death or last follow-up with the Kaplan-Meier method.

We intentionally included all LS available at the centres contributing to the data collection. This choice was taken to increase the number of LS in the biological analyses in order to better analyze the population of interest although it led to a sample with a LS proportion higher than the 12% observed at population-level. To test the association between the gene mutation and survival, we removed the bias of having a sample with a high proportion of LS readjusting their number. In other words, we decreased the number of LS in order to have a 12% of them. To this extend, we randomly selected LS simulating 100 different hypothetical samples. In each of this hypothetical sample, the NS were always all the patients of our sample whereas the LS were randomly selected from the LS in the database. To assess the association between mutated genes and OS, we applied the univariate Cox proportional hazard model to each sample and used Rubin's rule to pool the 100 estimates from these models (35).

To examine the association between *SMO* mutations and OS we applied Cox proportional hazard model adjusted for the available prognostic factors (sex, age, stage, histology and treatment) to each sample and used Rubin's rule to pool the 100 estimates from these models. P values (P) less than 0.05 were considered significant.

For the IHC analysis, the median semi-quantitative score was used as a good initial threshold to divide our sample into high and low SMO expression groups. To compare median survival across SMO expression groups in NS we used Laplace regression models for percentiles (36).

Results

The final analysis comprised 56 patients, 24 LS and 32 NS. No other LS with available tissue were found in our database.

Patients characteristics

Patients were mainly male, with epithelioid histotype, and diagnosed at stage III (*Table 1*). The clinical features did not differ significantly in the two groups. Out of the 29 patients who underwent surgery, the surgical approach was extra-pleural pneumonectomy in 20 cases, pleurectomy/decortication in 9 patients. The chemotherapy regimens, administered as neoadjuvant, adjuvant or first-line chemotherapy were platinum-based doublets in 39 out of 42 cases; the second drug was pemetrexed in 35 and gemcitabine in 4 patients. Three patients underwent monotherapy with pemetrexed. The systemic treatments administered as second or further lines were generally monotherapy with gemcitabine, vinorelbine, pemetrexed or treatments within clinical trials. Postoperative radiotherapy was performed in 23 patients; it was delivered to the ipsilateral hemithorax area in 18 patients and as prophylactic radiotherapy to chest wall tracts after surgery in 5 cases. Two patients, who did not undergo surgery, performed radiotherapy on the ipsilateral pleura. Palliative radiant treatments or palliative surgical approaches were not considered.

Table 1 Patients main characteristics overall and for NS and LS

Patients main characteristics	Subcategories	ALL (56 pts), N (%)	NS (32 pts), N (%)	LS (24 pts), N (%)	P (NS *vs.* LS)
Age, years	Mean	61.4	60.9	62.1	0.71
Sex	Female	15 [27]	8 [25]	7 [29]	0.77
	Male	41 [73]	24 [75]	17 [71]	
Histology	Epithelioid	51 [91]	29 [91]	22 [92]	0.36
	Mixed	4 [7]	3 [9]	1 [4]	
	Sarcomatoid	1 [2]	0 [0]	1 [4]	
Stage	I	5 [9]	3 [9]	2 [8]	0.42
	II	14 [25]	8 [25]	6 [25]	
	III	26 [46]	13 [41]	13 [54]	
	IV	10 [18]	8 [25]	2 [8]	
	Missing	1 [2]	0 [0]	1 [4]	
Radical surgery	Yes	29 [52]	16 [50]	13 [54]	0.76
	No	27 [48]	16 [50]	11 [46]	
Chemotherapy	Yes	42 [75]	23 [72]	19 [79]	0.76
	No	12 [21]	8 [25]	4 [17]	
	Missing	2 [4]	1 [3]	1 [4]	
Radiotherapy	Yes	25 [45]	11 [34]	14 [58]	0.10
	No	30 [54]	20 [63]	10 [42]	
	Missing	1 [2]	1 [3]	0 [0]	
Treatment	None	5 [9]	4 [13]	1 [4]	0.53
	One	22† [39]	13 [41]	9 [38]	
	Two	13‡ [23]	8 [25]	5 [21]	
	All three	16 [29]	7 [22]	9 [38]	

†, 18 chemotherapy, 3 surgery, 1 radiotherapy; ‡, 5 chemotherapy and surgery, 3 chemotherapy and radiotherapy, 5 surgery and radiotherapy. NS, normo-survivors, OS ≤3 years; LS, long survivors, OS >3 years.

Gene variations in the 21 MPM associated genes

Table 2 reports the sequencing analysis, with the mutated gene and the amino-acid residue involved. Eighteen out of 56 patients (32%) were wild-type for the genes analysed: 12 were NS and 6 were LS. The overall mutated genes and their distribution in NS and LS groups are reported in *Table 3*.

The only association between gene mutations and clinical features was *LATS2* and age: *LATS2* mutated patients were younger (43 *vs.* 62 years, P=0.01).

Mutations in at least five patients were observed only for *BAP1*, *NF2*, *TP53*, *SMO* and *PTCH1* genes. There were no significant differences in mutation frequency between the two groups (*Table 3*). Of note, *SMO* was mutated only in NS (15.6%) with epithelioid histotype (*Table 2*).

In NS patients the median survival was 21.6 (min 0.6; max 32.4) months, while in LS was 53 (min 37; max 86) months. To investigate the prognostic role of the more frequent mutated genes, we calculated the OS hazard ratio (HR) as reported in *Table 4*.

Only *SMO* mutational status was significantly associated with poor prognosis (P<0.0001) and remained a prognostic factor in multivariable analysis (HR =9.2, 95% CI: 3.0–28.4 P=0.0001). Kaplan-Meier curves show the prognostic role of *SMO*, considering that *SMO* mutated patients died

Table 2 Mutations across 56 MPM patients

MPM case	Histo type	Mutated genes	Survival group
1	E	*BAP1* (E600D)	LS
		TP53 (G187S)	
		KEAP1 (R596Q, A321V)	
2	E	*LATS2* (E505 stop)	NS
		NF2 (E421 stop)	
3	E	No mutations	NS
4	E	*PTCH1* (P725S)	LS
5	E	*TP53* (R248W)	NS
		KEAP1 (R554Q, E289K)	
		NF2 (R424C)	
6	E	*BAP1* (ins)	NS
7	E	No mutations	NS
8	E	*CDKN2A* (A148T)	NS
9	E	*NF2* (L436fs)	LS
10	E	*BAP1* (Q156 stop)	LS
11	E	No mutations	LS
12	E	*KDR* (T1038I)	NS
		SMO (R257Q)	
13	E	*BAP1* (R713Q)	NS
		SMO (T245M)	
		TP53 (R175H)	
		FBXW7 (R564C)	
14	E	*NF2* (I210T)	LS
15	E	No mutations	NS
16	E	*BAP1* (ins)	LS
17	E	No mutations	LS
18	E	No mutations	LS
19	E	*NF2* (R57STOP)	LS
20	E	*REV3L* (I691M)	LS
21	E	No mutations	LS
22	E	*BAP1* (K51fs)	NS
23	E	*PTCH1* (G1212S)	LS
24	E	*PTCH1* (K842R)	NS
		NF2 (R187fs)	
25	E	No mutations	NS
26	E	*NF2* (R341STOP)	NS
27	E	*KDR* (C482R)	LS
28	M	No mutations	NS
29	E	*TERT* (H815N)	LS
		TP53 (Y327 stop)	
30	E	No mutations	LS
31	E	*BAP1* (del)	NS
32	M	No mutations	NS
33	E	No mutations	NS
34	E	*TP53* (A119fs)	NS
35	E	*BAP1* (R699Q)	LS
		TERT (T292M)	
36	E	*BAP1* (R717W)	LS
37	E	*LATS2* (V212M)	NS
38	E	*BAP1* (E685 stop)	LS
39	E	*BAP1* (H193R)	NS
40	M	*NF2* (R196 stop)	NS
41	E	No mutations	LS
42	M	*NF2* (fs)	LS
		WT1 (513V)	
43	E	No mutations	NS
44	E	*TP53* (Y234C)	NS
45	E	No mutations	NS
46	S	*BAP1* (R610 stop)	LS
		TP53 (start loss M1K)	
47	E	*BAP1* (R60 stop)	LS
48	E	No mutations	NS
49	E	*TERT* (R1084 stop)	NS
		TP53 (R181C)	
50	E	*SMO* (R257W)	NS
		PTCH1 (G1363S)	
51	E	*SMO* (A601V)	NS
52	E	*PTCH1* (T1052M)	NS
53	E	No mutations	NS

Table 2 (*continued*)

Table 2 (*continued*)

MPM case	Histo type	Mutated genes	Survival group
54	E	No mutations	NS
55	E	*NF2* (E260 stop)	LS
56	E	*BAP1* (V447I)	NS
		FBXW7 (M269I)	

MPM, malignant pleural mesothelioma; E, epithelioid; M, mixed; S, sarcomatoid; NS, normo-survivors, OS ≤3 years; LS, long-survivors, OS >3 years.

Table 3 Distribution of gene mutations

Gene	All, N (%)	NS, N (%)	LS, N (%)	P (NS *vs.* LS)
Wild-type	18 (32.1)	12 (37.5)	6 (25.0)	0.243
BAP1	14 (25.0)	6 (18.8)	8 (33.3)	0.232
NF2	10 (17.9)	5 (15.6)	5 (20.8)	0.730
P53	8 (14.3)	5 (15.6)	3 (12.5)	0.686
SMO	5 (8.9)	5 (15.6)	0 (0.0)	0.063
PTCH1	5 (8.9)	3 (9.4)	2 (8.3)	1.000
TERT	3 (5.4)	1 (3.1)	2 (8.3)	0.571
FBXW7	2 (3.6)	2 (6.3)	0 (0.0)	0.501
LATS2	2 (3.6)	2 (6.3)	0 (0.0)	0.501
KDR	2 (3.6)	1 (3.1)	1 (4.2)	1.000
KEAP1	2 (3.6)	1 (3.1)	1 (4.2)	1.000
WT1	1 (1.8)	0 (0.0)	1 (4.2)	0.429
REV3L	1 (1.8)	0 (0.0)	1 (4.2)	0.429
CDKN2A	1 (1.8)	1 (3.1)	0 (0.0)	1.000

NS, normo-survivors, OS ≤3 years; LS, long survivors, OS >3 years. P values refer to the comparison between NS and LS.

within 13 months (*Figure 2*).

All the *SMO* mutations were missense and different. The same amino-acid residue was involved only in two patients, and the prediction of functional effects of all the variants was verified by PolyPhen-2 analysis (*Table 5*). Three mutations had never been described before, while mutation T245M was found in esophageal squamous cell carcinoma and mutation A601V in pancreatic cancer (37,38). *SMO* was mutated as a single gene or with mutations in other genes. We confirmed by Sanger the only mutation occurring with frequency higher than the limit of detection of this technique.

Table 4 Association between mutated genes in at least five patients and OS

Gene	HR (95% CI)	P
Any mutation-wild type	1.09 (0.71–1.67)	0.70
BAP1	1.14 (0.57–2.30)	0.73
NF2	1.16 (0.66–2.07)	0.60
TP53	0.91 (0.54–1.54)	0.36
SMO	4.36 (2.32–8.18)	<0.0001
PTCH1	1.16 (0.66–2.07)	0.71

OS is expressed as hazard ratio (HR) with 95% confidence interval (CI). Wild-type patients for the specific gene were used as reference to calculate P values.

SMO IHC

The expression of SMO was analyzed by IHC in 53 patients with leftover tissue to investigate the association between mutational status and the protein level (*Figure 3*). The median value of the semi quantitative score was 160 and divided the 53 patients into two groups: those with a high level of protein (≥160) and the others, with low expression (<160). The *SMO* mutated patients (4 of the 5 cases were available) all expressed a high level of protein. In addition, there were equal numbers of NS and LS (12 each) among the patients with low protein expression, while there were more NS with high expression (18 NS and 11 LS). The median survival of NS patients expressing high protein levels was about eight months less than those expressing low levels (11.6 *vs.* 19.6 months).

Discussion

We sequenced 56 MPM samples using a customized 21-gene panel. We confirmed that MPM has a low mutational burden and that *BAP1*, *NF2* and *TP53* are the most frequent mutated genes, as reported by other authors (20-25). For the first time, to our knowledge, we observed that *SMO* mutations were associated with a worse prognosis, with a HR of 9.2 (95% CI: 3.0–28.4, P=0.0001), although only 5 patients with *SMO* mutation were identified. We found *SMO* mutations in about 9% of our samples, and only in NS patients. We also found five *PTCH1* mutations, raising to 16% the overall frequency of alterations in the Hh pathway; however, these mutations did not show any clear correlation with survival. We evaluated patients managed in MPM expert centres in which interventional procedures,

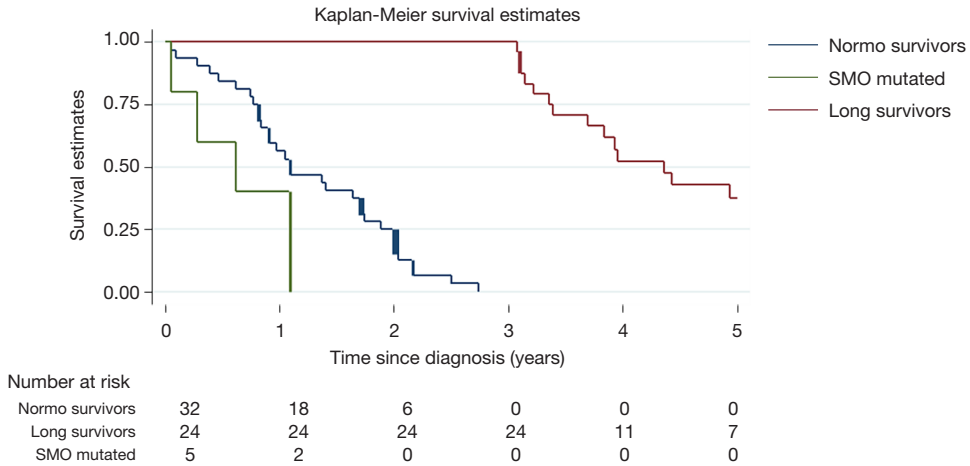

Figure 2 Kaplan-Meier survival estimates. Normo-survivors patients, OS ≤3 years, in blue; long survivors patients, OS >3 years, in red; SMO mutated patients in green.

Table 5 *SMO* mutations

SMO mutations (frequency)	Function prediction (polyphen)	Topology protein domain	Co-mutated genes
R257Q (16%)	Benign	1° cytoplasmic domain	*KDR*
T245M (11%)	Probably damaging	1° transmembrane domain	*BAP1, TP53, FBXW*
F484L (47%)	Probably damaging	4° extracellular domain	*BAP1*
R257W (9%)	Probably damaging	1° cytoplasmic domain	*PTCH1*
A601V (5%)	Probably damaging	4° cytoplasmic domain	–

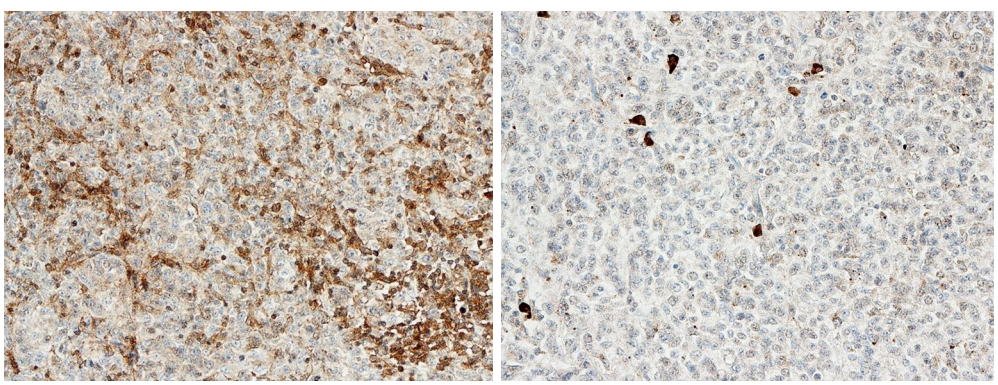

Figure 3 An example of high (on the left) and low (on the right) SMO expression level. IHC, 20×.

such as surgery, are more common, so that the rate of operated patients (52%) is higher than usual. However, we applied Cox proportional hazard model adjusted for the known prognostic factors (i.e., sex, age, stage, histology and treatment, including surgery) to assess the impact of *SMO* mutations on prognosis at the net of the available prognostic factors. In the past few years, some studies have shown that Hh signalling is active in MPM and regulates cell proliferation, in cell lines and clinical samples (32).

At preclinical level, Hh pathway was reported to be

up-regulated in MPM cancer cell lines with increased expression level of GLI1, GLI2, SMO, SHH, PTCH1 and PTCH2, that are indicative of an active Hh signaling. Zhang *et al*., in 46 MPM sample tissues, identified a correlation between higher SMO expression and worse survival (31). Non-synonymous mutation affecting *SMO*, *PTCH* and *SUFU* were detected, in both MPM cell lines and patients: the biological significance was unclear; however, the patient with the *SMO* insertion survived 3.4 months (39). At least six other studies have analyzed MPM mutations using NGS technology (20-25); the results were similar as regards the low mutational load and the most frequent mutated genes. However, the frequencies and the prognostic roles of each mutation differed across the studies, on account of their heterogeneity. All of them were retrospective and used different NGS approaches. Two studies reported a negative prognostic role for *TP53* mutations (22,23), which was not confirmed by De Rienzo or by this present work. No study showed a prognostic role of *BAP1* mutations although De Rienzo *et al*. found a correlation between higher *BAP1* expression and worse survival. In addition, deletions in *CDKN2A* were associated with poorer outcome (25). Our panel included this gene but it was not designed to detect large genetic losses (gene amplifications/losses and translocations). Consequently, we could describe only one *CDKN2A* missense mutation in our series.

Despite the methodological limits, all these studies have opened up some research areas in MPM. A proper prospective trial with adequate statistical power is warranted to define the true frequency of the reported mutations, their final prognostic role and the possibility of developing targeted treatments.

SMO and all the alterations of the Hh pathway might be potential therapeutic targets. In recent years, targeting Hh components has proved an interesting approach, at both preclinical and clinical levels (40). These compounds showed good activity *in vitro*, inhibiting the Hh downstream signaling and dramatically suppressing cell proliferation when used in range of nanomolar (41). Of note, they reduced tumor volume, although the majority of used models (mostly medulloblastoma, BCC and pancreas carcinoma) were not characterized for the mutational status of the Hh components. Vismodegib and sonidegib, two SMO inhibitors, have already been approved for the treatment of locally advanced BCC (42). In a rat MPM model, vismodegib gave good results in terms of tumor shrinkage and growth delay, with significant down-regulation of downstream transcriptional factor GLI1 in the stromal compartment (43). Other authors have reported suppression of cell growth in cell lines and animal models treated with different inhibitors directed against Hh components (44). Despite this preclinical evidence, however, in a phase I trial vismodegib failed to show any activity in the only three MPM patients included (45); however, these patients were not evaluated for alterations in the Hh pathway.

Although SMO protein expression was revealed by IHC in all our *SMO* mutated tumors, we still do not know whether these mutations are linked to a deregulated Hh pathway. We are therefore now working *in vitro* to clarify their final biological role.

Genomics has given us an important lesson on MPM including the discovery of a large number of wild-type patients which suggests that mechanisms different from activating mutations are implicated in this malignancy. Unfortunately, we are still far from finding effective strategies for this disease for which a wave of new cases is expected in many parts of the world. Thus, there is a pressing need for identification of new targets and therapeutic strategies for MPM.

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The present study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All the experimental protocols were approved by INT Independent Ethics Committee, code INT 91/13. Because the study was retrospective, the patients were not in treatment or in active follow-up; therefore, their informed consent was not required in accordance with Italian law (Gazzetta Ufficiale n. 72, 26/03/2012; n. 303, 29/12/2016).

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